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# Concordance of Macular Pigment Measurement Using Customized Heterochromatic Flicker Photometry and Fundus Autofluorescence in Age-Related Macular Degeneration

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**PURPOSE.** We compared macular pigment (MP) measurements using customized heterochromatic flicker photometry (Macular Metrics Densitometer) and dual-wavelength fundus autofluorescence (Heidelberg Spectralis HRA + OCT MultiColor) in subjects with early age-related macular degeneration (AMD).

**METHODS.** Macular pigment was measured in 117 subjects with early AMD (age, 44–88 years) using the Densitometer and Spectralis, as part of the Central Retinal Enrichment Supplementation Trial (CREST; ISRCTN13894787). Baseline and 6-month study visits data were used for the analyses. Agreement was investigated at four different retinal eccentricities, graphically and using indices of agreement, including Pearson correlation coefficient (precision), accuracy coefficient, and concordance correlation coefficient (ccc).

**RESULTS.** Agreement was poor between the Densitometer and Spectralis at all eccentricities, at baseline (e.g., at 0.25° eccentricity, accuracy = 0.63, precision = 0.35, ccc = 0.22) and at 6 months (e.g., at 0.25° eccentricity, accuracy = 0.52, precision = 0.43, ccc = 0.22). Agreement between the two devices was significantly greater for males at 0.5° and 1.0° of eccentricity. At all eccentricities, agreement was unaffected by cataract grade.

**CONCLUSIONS.** In subjects with early AMD, MP measurements obtained using the Densitometer and Spectralis are not statistically comparable and should not be used interchangeably in either the clinical or research setting. Despite this lack of agreement, statistically significant increases in MP, following 6 months of supplementation with macular carotenoids, were detected with each device, confirming that these devices are capable of measuring change in MP within subjects over time. (<http://www.controlled-trials.com/number/ISRCTN13894787>.)

**Keywords:** macular pigment, customized heterochromatic flicker photometry, fundus autofluorescence, age-related macular degeneration, concordance, agreement

Macular pigment (MP) is composed of the carotenoids, lutein (L), zeaxanthin (Z), and meso-zeaxanthin (MZ).<sup>1</sup> Macular pigment is found at the macula (the specialized part of the retina that mediates central vision) between the receptor axon and inner plexiform layers.<sup>2,3</sup> Macular pigment filters short-wavelength (blue) light and its constituent carotenoids have antioxidant<sup>4,5</sup> and anti-inflammatory properties.<sup>6–9</sup> Macular pigment's unique anatomic location, blue light filtering properties, and antioxidant and anti-inflammatory properties make this pigment important for visual function.<sup>10</sup> Macular pigment (and its constituent carotenoids) may have an important role in age-related macular degeneration (AMD) by reducing oxidative stress via its antioxidant properties as well as limiting the effect of inflammatory mediators in the pathogenesis of this condition.<sup>11</sup> Some studies also have reported that MP may be lower compared to controls among persons with glaucoma,<sup>12</sup> Alzheimer's disease (AD),<sup>13</sup> and diabetes,<sup>14,15</sup> suggesting that MP could be a useful biomarker for these conditions, providing a biologically plausible rationale to investigate whether MP has a role in these pathologies.

Given the importance of MP for visual function in diseased<sup>16–18</sup> and nondiseased retinas,<sup>19,20</sup> and the emerging evidence that MP may be a useful biomarker for AD,<sup>21,22</sup> researchers and clinicians must measure MP levels accurately across different populations. A variety of techniques are available for measuring MP (and its constituent carotenoids) and the measurement techniques can be classified broadly as ex vivo (i.e., outside cell/tissue) and in vivo techniques (i.e., inside cell/tissue). Ex vivo techniques include high performance liquid chromatography (HPLC) and microdensitometry. However, these ex vivo techniques can be performed only in postmortem eyes. In vivo techniques include physical techniques (e.g., dual-wavelength fundus autofluorescence (2WAF), fundus reflectometry, and raman spectroscopy) and psychophysical techniques (e.g., heterochromatic flicker photometry [HFP], customized heterochromatic flicker photometry [cHFP], color matching, and motion photometry), and these methods are desirable because they can be performed noninvasively in the living subject. The 2WAF technique also can be used ex vivo. Of note, however, there remains debate as to which

device, if any, should be deemed as “gold standard” for measuring MP. A review of the literature shows that HFP and cHFP are the most commonly used, but it is important to note that each device has its own advantages and limitations.

The Heidelberg Spectralis HRA+OCT MultiColor (Heidelberg Engineering GmbH, Heidelberg, Germany) is a new, commercially available device which uses the 2WAF technique to measure MP, whereas the Macular Densitometer (Macular Metrics, Corp., Providence, RI, USA) has been available for over a decade, with over 100 peer-reviewed scientific publications which have used this device. A previous study from our group compared MP measurements using the Spectralis to measurements obtained using the Densitometer (which uses cHFP), and reported good concordance between the results obtained by the two devices at four different retinal eccentricities.<sup>23</sup> However, that previous study was performed in subjects free of retinal disease with a mean age of  $49 \pm 13$  years.<sup>23</sup> Therefore, to our knowledge until now, concordance between the Spectralis and the Densitometer has not been evaluated appropriately in the AMD population. The current study was designed to investigate concordance between these two devices, and was done as part of the Central Retinal Enrichment Supplementation Trials (CREST),<sup>24</sup> a randomized double-blind clinical trial designed to investigate the impact of supplementation with the macular carotenoids (L, Z, and MZ) on visual function in healthy subjects with low MP and in subjects with early AMD (the study population used in the current investigation; CREST AMD [ISRCTN13894787]). We see this MP measurement concordance study as an important experiment, as it will inform researchers and clinicians on the agreement between the devices and emphasize the importance of not changing the measurement technique when assessing subjects/patients over time.

## METHODS

The design and methodology of the CREST study, including intervention assignment, has been described in detail previously.<sup>24</sup> For CREST AMD, the population of interest for the current investigation, the eligibility criteria included early AMD (one to eight on AREDS 11-step severity scale<sup>25</sup> in at least one eye [the study eye], confirmed by the Moorfields Eye Hospital Reading Centre [MEHRC], London, UK), best-corrected visual acuity (BCVA) of 6/12 (20/40) or better, no more than five diopters spherical equivalence of refraction, no previous consumption of supplements containing the macular carotenoids (L, Z, and/or MZ), no other retinal pathology beyond AMD, and no diabetes mellitus (by self-report). Ethical approval was granted by Research Ethics Committee of the Waterford Institute of Technology (WIT), Waterford, Ireland, and the Ethics Committee of the European Research Council (ERC). Written informed consent was obtained from each subject before study enrollment. The tenets of the Declaration of Helsinki were followed in the experimental procedures. A comprehensive clinical assessment, which included MP measurement using cHFP and 2WAF (see below), was conducted at the Macular Pigment Research Group (available in the public domain at [www.mprg.ie](http://www.mprg.ie)), Vision Research Centre, Waterford Institute of Technology, Ireland by the study investigator (KOA), who was trained in all aspects of the CREST protocol. All subjects for this investigation were naïve to the MP measurement protocols and had no previous experience with any of the tests.

## Macular Pigment Measurement

**MP Measurement by cHFP.** Macular pigment was measured by cHFP using the Macular Densitometer (Macular

Metrics, Corp.) at 0.25°, 0.5°, 1.0°, and 1.75° of retinal eccentricity, with a reference point at 7°. This protocol has been described in detail previously and has been validated in AMD subjects by comparing the *in vivo* spectral absorption curves from this device to the *ex vivo* spectral absorption curves of the macular carotenoids.<sup>27</sup> Two wavelengths of light, one at 458 nm (blue light; wavelength that is well absorbed by MP) and the other at 550 nm (green light; wavelength that is not absorbed by MP) are arranged in a stimulus-surround configuration where the stimulus consists of a target presented in counterphase flicker (alternating blue to green). The blue and green alternating lights are inverse-yoked so that when the blue light is adjusted to be more intense, the green light is decreased commensurately and vice versa. The radiance of the blue and green lights are adjusted by turning a dial until the flicker of the disk stops (null flicker) or it is at a point of minimal flicker. Thus, null flicker occurs when there is isoluminance of the blue and green lights.

Before MP measurements, the testing procedure was explained and the subject's critical flicker frequency (CFF) was estimated using a prediction table based on age. Setting the flicker rate according to an expected optimal for a narrow null zone helps to minimize the variance in radiance values obtained during MP measurements at a given retinal eccentricity. If the subject could not reach the null flicker, the CFF was increased by 1 Hz in a stepwise fashion until null flicker was perceived. Also, if the subject exhibited a wide variation in null flicker reading, the CFF was decreased in steps of 1 Hz until an acceptable null range was achieved. During the test, subjects were instructed to turn the radiance knob clockwise or counterclockwise until the flickering stops or it is at a point of minimal flicker. The starting radiance is alternated, so that the knob is not always turned in the same direction. Throughout the testing, subjects were reminded to blink, and instructions were repeated where necessary. Six measurements at each of the targets (0.25°, 0.5°, 1°, 1.75°, and a reference point at 7°) were taken for each subject. The MP measurement at a specified retinal eccentricity was computed from the radiance values obtained where the subject reported null flicker and these radiance values were deemed reliable and acceptable only when the standard deviation of the MP value was below 0.1 optical density units (ODU). The log ratio of the difference in radiance values between the measurement at a particular retinal eccentricity (0.25°, 0.5°, 1°, 1.75°) and the measurement at 7° yields the MP optical density at the specified test locus. Data were not obtained from three (2.6%) subjects because they could not complete the test reliably.

**Pupillary Dilation.** Pupils were dilated using a drop each of 0.5% proxymetacaine hydrochloride, 2.5% phenylephrine hydrochloride, and 1% tropicamide before performing MP measurement by 2WAF, retinal photography, and cataract grading.

**MP Measurement by 2WAF.** Macular pigment was measured by 2WAF<sup>28–30</sup> using the Spectralis HRA+OCT MultiColor (Heidelberg Engineering GmbH). This new technology uses confocal scanning laser ophthalmoscopy imaging with diode lasers to measure MP.<sup>23,31</sup> The 2WAF technique works on the principle of excitation of fluorophores (primarily lipofuscin) in the retina and provides a single-pass MP measurement. Fluorescence is an internal property of certain molecules (known as fluorophores) which makes them emit light when excited at certain wavelengths. Lipofuscin is excited by light between 400 and 590 nm and emits light between 520 and 800 nm.<sup>32,33</sup> The excitation spectrum of lipofuscin overlaps with the absorption spectrum of MP (400–550 nm with maximum absorption at 460 nm)<sup>2</sup> and this property is used in the 2WAF technique.

**TABLE 1.** Demographic, Lifestyle, Cataract, and AMD Characteristics of Subjects Included in This Report

Variable	
Age, y, mean $\pm$ SD	64.68 $\pm$ 9.08
Sex, <i>n</i> (%)	
Male	39 (33.7)
Female	78 (66.7)
Cataract, mean $\pm$ SD*	
Nuclear opalescence	1.65 $\pm$ 0.83
Nuclear color	2.40 $\pm$ 0.95
Cortical	0.84 $\pm$ 1.03
Posterior subcapsular	0.35 $\pm$ 0.56
Pseudophakia, <i>n</i> (%)	6 (5.1)
AMD grades, <i>n</i> (%)	
1–3	30 (25.6)
4–5	57 (48.7)
6–8	30 (25.6)
SP_MP, mean $\pm$ SD*	
0.23	0.52 $\pm$ 0.19
0.47	0.44 $\pm$ 0.17
0.98	0.33 $\pm$ 0.14
1.76	0.14 $\pm$ 0.08
DM_MP, mean $\pm$ SD*	
0.25	0.75 $\pm$ 0.26
0.5	0.63 $\pm$ 0.22
1	0.45 $\pm$ 0.17
1.75	0.32 $\pm$ 0.14

Data displayed are mean  $\pm$  SD for interval data and percentages for categorical data. SP\_MP, macular pigment at 0.23°, 0.47°, 0.98°, and 1.76° eccentricity using dual-wavelength fundus autofluorescence (Heidelberg Spectralis HRA + OCT MultiColor); DM\_MP, macular pigment at 0.25°, 0.5°, 1.0° and 1.75° eccentricity using customized heterochromatic flicker photometry (cHFP; Macular Metrics Densitometer). Age-related macular degeneration grades are based on the AREDS 11-step severity scale.

\* *n* = 117 as certain tests/measures could not be obtained.

Before MP measurement, alignment, focus and illumination are first adjusted in infrared mode. Once the image is illuminated evenly, the laser mode is switched from infrared to blue plus green laser light autofluorescence. Focus and illumination are readjusted for optimal acquisition. The retina is illuminated simultaneously with two wavelengths of light (486 nm, which is absorbed by MP, and 518 nm, which is not well absorbed by MP) to obtain a series of autofluorescence images within 30 seconds. These images are digitally subtracted to generate MP spatial distribution maps with the reference set at 7°. Macular pigment at 0.23°, 0.47°, 0.98°, and 1.76° retinal eccentricities was recorded. Data were not obtained in two (1.7%) subjects because one subject found bright lights unbearable and refused to continue the test, and the other subject was not able to open the eyes sufficiently for a reliable measurement to be obtained.

### Retinal Photography and AMD Grading

Stereoscopic color fundus photographs (45°) were taken in three retinal photographic fields (optic disc, macula, temporal to macula) using a Zeiss Visucam 200 (Carl Zeiss Meditec AG, Jena, Germany). In addition, a monoscopic photograph of the anterior segment was taken to document any media opacities. Photographs were transferred to the MEHRC, London, UK via an encrypted system and were graded in a masked fashion adhering to the Age-Related Eye Disease Study (AREDS) 11-step severity scale.<sup>25</sup>

### Cataract Grading

Cataract grading was performed using the Haag-Streit BM 900 slit-lamp biomicroscope (Haag-Streit AG, Koeniz, Switzerland) adhering to the Lens Opacities Classification System III (LOCS III)<sup>34</sup> within the first year of this study by a trained and certified grader (KOA).

The degree of nuclear opalescence (NO) and color (NC) was graded on a scale ranging from 0.1 to 6.9 while cortical (C) and posterior subcapsular (PSC) opacities were graded on a scale ranging from 0.1 to 5.9. In addition, pseudophakia versus cataract in the study eye also was recorded during grading.

### Statistical Analysis

One eye (the study eye) of each subject comprised the unit of analysis. The study eye was chosen by adhering to the eligibility criteria with particular emphasis on the presence of early AMD, BCVA of 6/12 (20/40), no more than five diopters spherical equivalence of refraction and no other retinal pathology beyond AMD. The study eye could be either the right or left eye. If both eyes had early AMD, the eye with the best BCVA was chosen as the study eye. However, if both eyes had the same BCVA, the right eye was selected. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 22.0 (IBM, Armonk, NY). Data analyzed included baseline and 6-month study visits. We decided to use 6-month data, in addition to baseline data, for the following reasons: (1) to investigate whether agreement between the two devices was the same at two different time points (in other words, is there better agreement [as determined by agreement indices] at 6 months in comparison to baseline?); and (2) to investigate whether the two devices are able to consistently detect changes in MP following 6 months of supplementation with the macular carotenoids. Of note, 6-month data should represent increased MP levels in all subjects, given that all subjects in CREST AMD were consuming a formulation containing either 10 mg/day L, 10 mg/day MZ, and 2 mg/day Z or 10 mg/day L and 2 mg/day Z.<sup>24</sup> Uniquely, this allows us to assess concordance at baseline and at 6 months, and also the capacity of each device to detect change in MP over time. Agreement indices, and confidence limits for these indices, were obtained using the statistical programming language R code<sup>35</sup> supplied with Lin et al.<sup>36</sup> Agreement was investigated graphically using ordinary scatterplots of MP from the two devices being compared (with line  $y = x$  superimposed). In addition, agreement was assessed using three indices of agreement (see Appendix): (1) precision, the Pearson correlation coefficient, measures the degree of scatter, with values close to 1 indicating closeness to the ordinary least squares regression line (and, hence, little scatter); (2) accuracy, constructed from the means and standard deviations of the two variables being compared, with values close to 1 indicating that the two means are close to each other and that the two standard deviations are close to each other; and (3) concordance correlation coefficient (ccc), obtained as the product of the other two coefficients.

The possible effect on agreement of age, sex, AMD, and cataract, was investigated using a general linear model. Level of significance was set at  $P < 0.05$  without adjusting for multiple comparisons.

### RESULTS

Table 1 presents the demographic, MP, cataract, and AMD grades of all subjects included in this report. Agreement indices between the Densitometer and Spectralis at baseline study visit are shown in Table 2. The Figure shows the scatterplots of the



**TABLE 2.** Agreement Indices for MP Measurements Using the Macular Densitometer and the Heidelberg Spectralis HRA+OCT Multicolor at Baseline (V1)

Measure	ccc	Precision	Accuracy
0.25	0.22 (0.12)	0.35 (0.21)	0.63 (0.54)
0.5	0.27 (0.17)	0.41 (0.27)	0.67 (0.58)
1.0	0.20 (0.08)	0.26 (0.11)	0.76 (0.66)
1.75	0.08 (0.01)	0.19 (0.04)	0.41 (0.34)

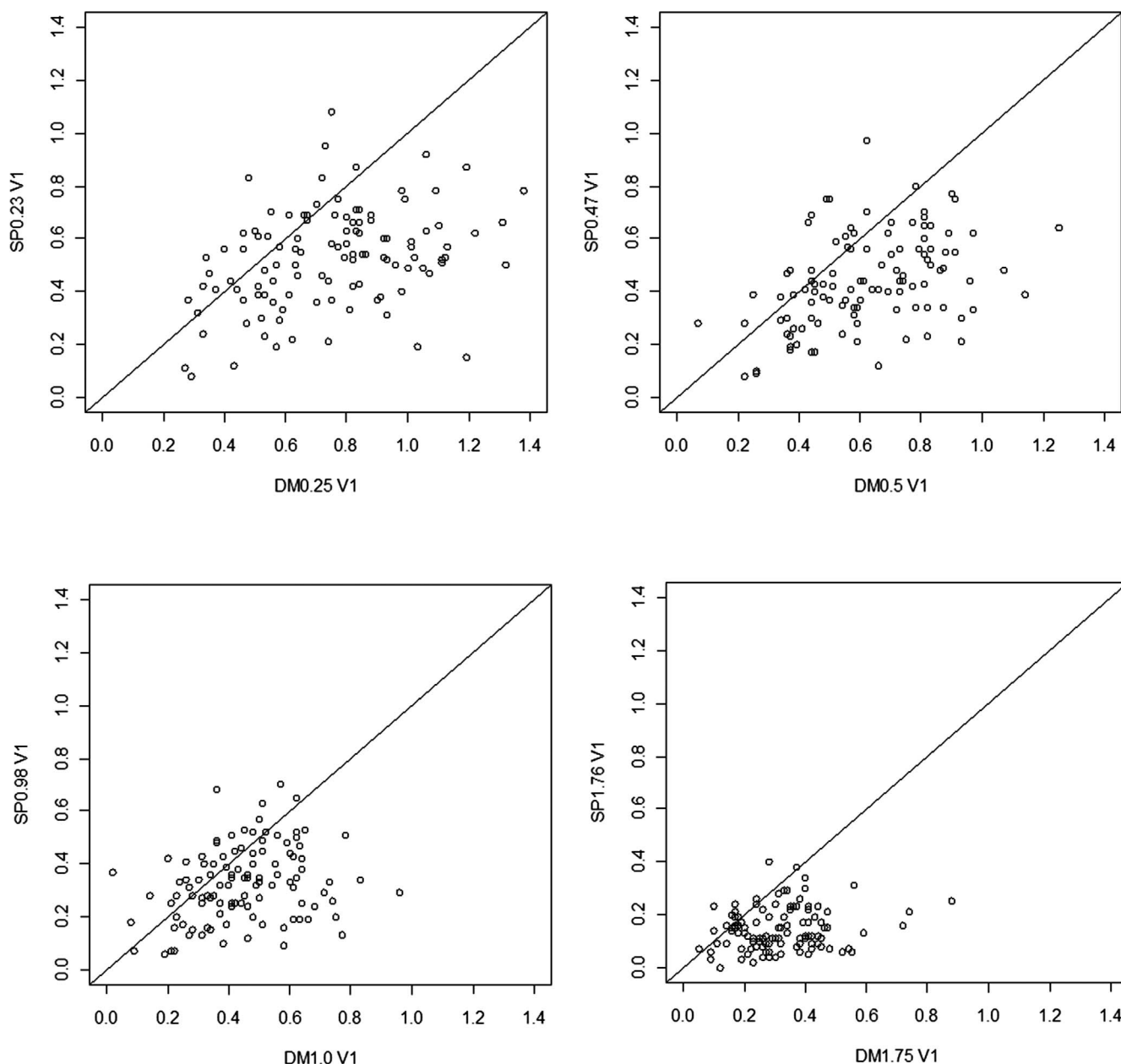
For each coefficient, the 95% lower confidence limit is shown in parentheses.

MP values at 0.25°, 0.50°, 1.00°, and 1.75° eccentricity, with the line  $y = x$  superimposed. Table 2 and the Figure exhibit poor agreement between the two devices at all eccentricities.

We investigated the effect on agreement of other study variables, using general linear models with difference in measured MP (Densitometer-Spectralis) as dependent variable.

### Effect of Age and Sex on Agreement Between the Two Devices

The effect of age and sex on agreement at the four retinal eccentricities was investigated using a general linear model. Agreement was unaffected by age ( $P > 0.05$ , for all eccentricities). However, disagreement between the two



**FIGURE.** Scatterplots with the line  $y = x$  superimposed comparing macular pigment measurements using the Macular Densitometer (DM) to the Heidelberg Spectralis HRA+OCT Multicolor (SP) at baseline (V1).

**TABLE 3.** Subgroup Analyses of the Average Differences in MP Measurements Using the Macular Densitometer and the Heidelberg Spectralis HRA+OCT Multicolor at Baseline (V1)

Subgroups	<i>n</i>	MP at 0.25		MP at 0.5		MP at 1.0		MP at 1.75	
		Av. Diff., DM-SP	Sig.	Av. Diff., DM-SP	Sig.	Av. Diff., DM-SP	Sig.	Av. Diff., DM-SP	Sig.
Age, y									
<65	61	0.25	0.302	0.22	0.093	0.16	0.029	0.19	0.161
≥65	56	0.20		0.15		0.08		0.15	
Sex									
Male	36	0.27	0.164	0.25	0.025	0.19	0.007	0.20	0.112
Female	76	0.20		0.16		0.09		0.15	
AMD grades									
1–3	28	0.26	0.356	0.23	0.248	0.19	0.019	0.21	0.111
4–8	84	0.21		0.17		0.10		0.16	
Cataract grades*									
Nuclear opalescence									
<2.5	83	0.24	0.805	0.20	0.844	0.13	0.834	0.18	0.529
≥2.5	7	0.27		0.22		0.11		0.21	
Nuclear Color									
<2.5	55	0.23	0.621	0.18	0.329	0.12	0.829	0.16	0.177
≥2.5	35	0.26		0.23		0.13		0.21	
Cortical									
<1.0	60	0.25	0.678	0.22	0.360	0.13	0.727	0.18	0.830
≥1.0	30	0.23		0.17		0.12		0.18	
Posterior subcapsular									
<0.5	74	0.25	0.688	0.21	0.557	0.14	0.135	0.19	0.053
≥0.5	16	0.22		0.17		0.06		0.11	

Age-related macular degeneration grades are based on the AREDS 11-step severity scale. *n*, number; Av. Diff., DM-SP, average differences in macular pigment between the Densitometer (Macular Metrics Densitometer) and the Spectralis (Heidelberg Spectralis HRA + OCT MultiColor); Sig., level of significance set at standard  $P < 0.05$  and obtained using the Independent samples *t*-test.

\*  $n \neq 117$  as certain tests/measures could not be obtained.

devices was significantly greater for males at 0.5° ( $P = 0.025$ ) and 1.0° ( $P = 0.007$ ) of eccentricity.

### Effect of Cataract Grade and Pseudophakia on Agreement Between the Two Devices

The effect of cataract grades (NO, NC, C, PSC) on agreement between the two devices was investigated using a general linear model, independently and after controlling for age and sex. Agreement was unaffected by grade of cataract in all our analyses ( $P > 0.05$ ). The effect of pseudophakia on agreement also was investigated using a general linear model, but the nonsignificant result in this case may be due to lack of statistical power, as there were few cases of pseudophakia in this study (see Table 1).

### Effect of AMD Grade on Agreement

The effect of AMD grades (as defined on the AREDS 11-step severity scale) on agreement between the two devices was investigated using a general linear model, independently and after controlling for age and sex. We combined AMD grades 1 to 3 and 4 to 8 for this analysis. Agreement was unaffected by AMD grade in all general linear model analyses controlling for age and sex ( $P > 0.05$ , for all). Not controlling for age and sex, there is a relationship between AMD grade and disagreement at 1.0° eccentricity ( $P = 0.019$ ), but greater disagreement occurs in the low-risk (1–3) AMD category (Table 3).

### Agreement Within Study Subgroups

These findings (on the relationship between agreement and other study variables) are summarized in Table 3, using simple binary classifications of age and cataract grades. The *P* values displayed in Table 3 are from the independent samples *t*-test, and there is one more statistically significant result than from the earlier general linear model analysis; however, greater disagreement in Table 3 is found among the under-65s, not the older subjects.

### Is Agreement Different Between Study Visits, That Is, Baseline (V1) and 6 Months (V2)?

The agreement indices at baseline and 6 months were similar; that is, there still is, at 6 months, poor agreement between the Densitometer and Spectralis at all retinal eccentricities (e.g., baseline at 0.25° eccentricity, accuracy = 0.63, precision = 0.35, ccc = 0.22; 6 months at 0.25° eccentricity, accuracy = 0.52, precision = 0.43, ccc = 0.22).

### Measuring Change in MP Over Time

Despite these differences between MP measured by the two devices, when we look at change in MP over time (baseline versus 6-month study visit), using a paired *t*-test, the conclusion is the same; that is, on average, MP increases significantly after 6 months of supplementation, whichever

device is used. For example, mean MP at 0.25° eccentricity measured on the Densitometer increases from 0.76 to 0.85 (12%) after 6 months ( $P < 0.001$ ), and mean MP at 0.23° eccentricity measured on Spectralis increases from 0.52 to 0.60 (15%;  $P < 0.001$ ).

## DISCUSSION

In the present study, we evaluated the concordance of two MP measuring devices (Densitometer and Spectralis) in subjects with early AMD, and assessed the ability of these devices to detect change in MP over time. In brief, the results of this experiment suggested that MP measurement on both devices are not statistically comparable. Assessing the data (see Fig.), it appears that the Spectralis tends to undervalue MP measurements when compared to the Densitometer. Moreover, this poor agreement was not only present at baseline (before supplementation with the macular carotenoids), but it also was seen at 6 months following supplementation with the macular carotenoids. However, it is important to point out that both devices were capable of detecting changes within subjects following supplementation with formulations containing the macular carotenoids.

Of note, the Spectralis device used in this study has been compared previously (by our group) with the Densitometer in normal healthy subjects.<sup>23</sup> In contrast to the current study, concordance in subjects free of retinal disease was good.<sup>23</sup>

Possible reasons for the lack of agreement in the current study are discussed. Firstly, we studied factors that we believe may contribute to the poor concordance we identified between the two devices in the early AMD population. The variables examined included age, sex, cataract, and AMD grade, and the analysis was done with general linear models (i.e., controlling for other variables) and based on binary versions of each variable (older and younger, lower and higher risk of AMD, and so forth). Of note, we found that only sex was associated consistently with disagreement, at 0.5° and 1.0° eccentricities, with greater disagreement between devices for male subjects. Age and AMD grades were related to disagreement at 1.0° eccentricity, but only in the simplified binary analysis (Table 3), and in both cases the finding was counterintuitive, with younger and lower-risk subjects exhibiting greater disagreement. Given that we did not adjust for multiple testing in this study, these reported significant differences in agreement (for age, sex, and AMD status) should be treated circumspectly: they may be the result of Type I errors.

Of note, MP measurement by chFP has been shown to be unaffected by cataracts.<sup>37,38</sup> However, it may be surprising that cataract grade did not explain, at least in part, the lack of agreement we observed between the two devices, given that MP measurements by fundus autofluorescence have been reported previously to be affected by cataracts.<sup>39,40</sup> It is important to note, however, that these previous studies, testing the impact of cataract on MP measurement using fundus autofluorescence, used different hardware and software to that used by the Spectralis. Also, in our study, cataract grading was conducted within the first year of the study, rather than at baseline, and it is possible, but unlikely, that this may have influenced our results. Also, it is important to point out that the level of cataract in our study population was not severe (e.g., mean NO 1.65; see Table 1), and it is likely that if cataract is to impact on MP measurement when using fundus autofluorescence, that this would be directly related to severity grade of cataract.<sup>39,40</sup> Finally, and most importantly, this study was not designed to investigate the impact of cataract on Spectralis 2WAF MP measurement. Indeed, a study sufficiently

powered with appropriate design to investigate the influence of cataract on Spectralis 2WAF MP measurement would involve MP measurements before and after cataract surgery, and this precise experiment currently is underway at our research center.

Fundamentally, the two devices work on different principles to obtain measurements of MP, with many different assumptions inherent in each device. For example, chFP requires subjects to follow instructions and to make decisions to obtain MP readings, whereas the 2WAF method does not require subjects' responses or decision-making to obtain MP readings. The subject simply is required to fixate on an internal target within the system for circa 30 seconds. Therefore, MP is measured quickly using the Spectralis, and subject fatigue is not an issue, whereas with the Densitometer, MP measurement takes circa 30 minutes to obtain MP data at four different retinal eccentricities. During chFP, Troxler's fading may be induced when viewing the target at the peripheral reference locus (7° eccentricity), which is a limitation of this technique. The Spectralis, however, does require pupil dilation, and some subjects do find the bright lights used in the Spectralis uncomfortable. Nevertheless, the Spectralis is a class 1 laser device, which complies with all applicable international standards with respect to safety. Interestingly, the poor concordance between the devices also was seen at 6 months following supplementation with the macular carotenoids, ruling out any possible learning effect with MP measurement using either device. The lack of concordance may be explained, at least in part, by the following assumptions of the 2WAF method: (1) no fluorophores anterior to MP, which cannot be compensated for by digital subtraction of the two autofluorescence images, and (2) the type and composition of fluorophores is assumed to be constant.<sup>28,31,41</sup> The assumption that lipofuscin is equally distributed has been suggested to be compensated by the use of two wavelengths.<sup>28,31</sup>

Despite the lack of agreement between the two devices, statistically significant increases in MP, following 6 months of supplementation with macular carotenoids, were recorded using each device. This finding suggests that, for longitudinal analyses, if the requirement is to detect change over time, both devices are capable of detecting such change (i.e., following supplementation with the macular carotenoids). An important finding from our study is that it is not appropriate to switch between devices when measuring MP in the same subjects over time. For example, mean MP at 0.25° eccentricity, in our study, was 0.76 ODU on the Densitometer at baseline, whereas at 6 months mean MP at 0.23° eccentricity was 0.60 ODU on the Spectralis. Comparison of these two results (baseline for Densitometer and 6 months for Spectralis) would suggest a decrease of 21% in average MP after 6 months of macular carotenoid supplementation in AMD subjects, which clearly is not the case, as a significant and comparable increase was seen with each instrument in subjects over time (12% at 0.25° for Densitometer and 15% at 0.23° for Spectralis).

Each device, therefore, may be suited to a given population, and perhaps more appropriate for a particular research setting. It is important that each device is underpinned by its normative database, given the current diversity of MP research, and the need to measure MP accurately in the research and clinic setting. In summary, for clinical or research purposes, it is advisable that the same device be used for baseline and follow-up visits within a given study.

The strengths of this study include the following: (1) MP measurements were conducted in a relatively large sample ( $n = 117$ ) of subjects with early AMD at two time points (baseline and 6 months); (2) assessment of AMD morphology was performed by an accredited reading center in a masked fashion; (3) cataract grading was conducted by a trained and

certified LOCS III grader; (4) subjects were naïve to both tests, thereby limiting potential confounding attributable to subject bias; and (5) one trained examiner conducted MP measurements on both devices; therefore, eliminating interexaminer bias and variability. The main limitation of this study is that cataract grades were obtained within the first year of the study rather than at baseline.

In conclusion, specific MP values obtained using the Spectralis are not comparable to MP values obtained using the Densitometer in subjects with early AMD. These two devices should not, for AMD subjects, be used interchangeably in the clinical or research settings, but each device is capable of reliably detecting and quantifying change in MP following supplementation with MP's constituent carotenoids. Accordingly, it is advisable that the same device be used within a given study. Furthermore, each of these two devices should be underpinned by its respective and separate normative database.

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## APPENDIX: EXPLANATION OF AGREEMENT INDICES

Notation: Variables  $X$  and  $Y$ , means  $\text{Mean}(X)$  and  $\text{Mean}(Y)$ , standard deviations  $\text{SD}(X)$  and  $\text{SD}(Y)$ , and covariance  $\text{Cov}(X, Y)$ .

$$\text{Precision} = \frac{\text{Cov}(X, Y)}{\text{SD}(X) * \text{SD}(Y)} \quad (1)$$

Precision is the ordinary Pearson correlation coefficient, and measures the degree of scatter in the  $(X, Y)$  plot around the best-fitting regression line.

$$\text{Accuracy} = \frac{2}{w + \frac{1}{w} + v^2}, \quad (2)$$

where

$$w = \frac{\text{SD}(X)}{\text{SD}(Y)} \quad \text{and} \quad v = \frac{\text{Mean}(Y) - \text{Mean}(X)}{\sqrt{\text{SD}(X) * \text{SD}(Y)}}.$$

Accuracy will be close to 1 if the two means are close in value and the two standard deviations are close in value.

$$\text{Concordance} = \text{Precision} * \text{Accuracy} \quad (3)$$

Concordance will be close to 1 if precision and accuracy are both close to 1. Whereas the Pearson correlation coefficient measures degree of scatter around the least squares regression line, the ccc measures degree of scatter around the line  $y = x$ , and for this reason is a better measure of agreement.